(54) Title: STABLE TRANSFORMATION of UNDIFFERENTIATED CONIFER CELLS

(57) Abstract

The invention provides a technique for stably inserting foreign genes into conifer cells. The cells to be transformed are in an undifferentiated state such that embryos are likely to regenerate from a single cell. Because of the use of this approach, the probability that the stably transformed plants will be chimaeric is low or non-existent. Conifer embryogenic tissue may be transformed subjected to extremely toxic levels of selection agent after insertion of foreign genes wherein the foreign genes include genes protective against the selection agent and wherein only stably transformed embryogenic tissue grows on the medium, and non-transformed tissue does not grow. The selected tissue may be used to produce transgenic plants. In preferred embodiments the cells to be transformed are maintained on a medium having concentrations of inorganic ions within a prescribed range.

STABLE TRANSFORMATION OF UNDIFFERENTIATED CONIFER CELLS.

TECHNICAL FIELD

This invention relates to a method of inserting foreign genes into conifers.

5   BACKGROUND ART

Genetic engineering has already been successfully applied to various annual crops with economic importance, and numerous genetically engineered crops are grown commercially or are in the process of field testing worldwide. Transformation of conifer tissue has been demonstrated using *Agrobacterium tumefaciens*-mediated gene transfer and transgenic plants

10   have occasionally been regenerated (Huang et al, 1991)[1]. However, conifers have proven to be much less susceptible to *Agrobacterium* infection than numerous crop plants. For this reason other types of gene transfer protocols have been explored with conifers. For example, Wilson et al (1989)[2] studied PEG-mediated expression of *gus* and *cat* genes in protoplasts from embryogenic suspension cultures of *Picea glauca*. Bekkaouni et al (1988)[3]

15   and Tautorus et al (1989)[4] studied transient gene expression in electroporated protoplasts from conifer species. Walter et al (1994)[5] reported the transient expression of the *gus* gene controlled by different monocotyledonous and dicotyledonous promoters in *Pinus radiata* embryogenic tissue.

20   Certain conifer species are important commercially especially for production of timber and pulp and paper. Open pollinated and control pollinated seed orchard technologies have been used to obtain major improvements in growth and form of these conifers. However, the use of genetic transformation with a view to obtaining rapid improvement or the addition of new traits has considerable commercial potential.

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It is an object of the present invention to provide a method for inserting foreign genes into conifer species in such a manner as to give a good prospect of regenerating stably transformed plants. At the least, it is an object to provide the public with a useful choice.

5   SUMMARY OF THE INVENTION

According to one aspect of the invention, there is provided a technique for stably inserting foreign genes into conifer cells wherein the cells to be transformed are in an undifferentiated state such that embryos are likely to regenerate from a single cell. Because
10   of the use of this approach, the probability that the stably transformed plants will be chimaeric is low or non-existent.

In another aspect the invention provides a method of producing transgenic plants. Conifer embryogenic tissue is transformed and the transformed tissue is subjected to extremely toxic
15   levels of selection agent after insertion of foreign genes wherein the foreign genes include genes protective against the selection agent and wherein only stably transformed embryogenic tissue grows on the medium, and non-transformed tissue does not grow. The selected tissue is used to produce transgenic plants.

20   DETAILED DESCRIPTION OF THE INVENTION

The invention relates to methods for inserting foreign genes into conifers and regenerating stably transformed plants.

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The tissue to be transformed consists of cells in an undifferentiated state such that embryos are likely to regenerate from a single cell. By using this approach the probability that the stably transformed plants produced will be chimaeric is low or non-existent.

5   Methods for capturing embryogenic tissue from conifers and using it to generate embryos and subsequently coniferous plants have been described in Australian Patent Application 60707/94. These methods are preferred, but other methods for producing plants by embryogenesis may be used. Preferably the embryogenic material to be transformed is tissue maintained so that it never develops past the 8-cell stage on standard embryogenesis

10   medium (Australian Patent Application 60707/94).

Alternatively, the tissue may be maintained on embryo development medium (Australian Patent Application 60707/94) modified in that it contains 2,4-D and/or 6-benzylaminopurine. These hormones are included to favour proliferation of tissue rather

15   than development. Preferably both hormones are present with the 2,4-D being present at 1-5 mg/l and the benzylaminopurine being present at 0.1 - 0.9 mg/l. Other media are also suitable.

Table 1 column A lists inorganic ion concentrations which are preferred for at least one of

20   (preferably all of) maintenance of embryogenic tissue before transformation, maintenance during transformation, maintenance after transformation and also development and maturation of the conifer embryos. Those of column B in the same table are more preferred and those in column C of Table 1 are most preferred.

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TABLE 1

MEDIA ION CONCENTRATIONS
(mmoles/l)

ION	A	B	C
NO_3	8-27	13-23	17.8
NH_4	0.95-3	1.5-2.5	1.96
Ca	0.08-0.25	0.12-0.21	0.17
Fe	0.05-0.15	0.07-0.13	0.10
Na	1.9-5.75	2.9-4.9	3.85
Zn	0.045-0.135	0.06-0.12	0.09
Cu	$4.5 \times 10^{-3} - 1.5 \times 10^{-2}$	$7 \times 10^{-3} - 1.2 \times 10^{-2}$	9.61×10^{-3}
Mg	0.8-2.5	1.2-2.0	1.62

Potassium, chloride, phosphate, manganese, borate, sulphate, iodide, molybdenum, and cobalt ions are preferably included in the medium. Particularly preferred are the inorganic ion concentrations shown in Table 2.

TABLE 2

MEDIA ION CONCENTRATIONS

ION	Concentration (mmoles/l)
NO_3	17.8
NH_4	1.96
P	1.96
K	14.16
Ca	0.17
Mg	1.62
Cl	3.42×10^{-1}
Fe	0.10
S	1.83
Na	3.85
B	0.13
Mn	1.62×10^{-2}
Zn	0.09
Cu	9.61×10^{-3}
Mo	8.27×10^{-4}
Co	8.41×10^{-4}
I	6.02×10^{-3}

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The media used in the invention preferably also contain other nutrients generally used in conifer embryogenesis such as a carbon source. In the preferred media vitamins especially thiamine, nicotinic acid, and pyridoxine are preferably also included. Inositol, sucrose, agar and glutamine and other amino acids (particularly asparagine, arginine, citrulline, ornithine,

5   lysine, alanine and proline and preferably all of these) are also preferably present in preferred media. The concentrations of some of the other components can usefully be varied when embryo development and maturation is required. For example it is preferred to include glutamine and other amino acids in relatively high concentrations for the later stages of embyrogenesis. Also preferred for use in the invention are media having

10   concentrations of the nondistinguishing components at levels between 50% and 150% of those found in Tables A5 and A6 (Example 3). Highly preferred are media with concentrations of the non-distinguishing components at 75% to 125% of those found in the solutions of Tables A5 and A6. For media resembling that in Table A6, 2,4-D and/or 6-benzylaminopurine may be necessary to favour proliferation rather than development. The

15   preferred pH for media in the range 5.5-5.9. Agar may be included in preferred media at 0.5-1.5g/l (w/v). Sucrose may be included in preferred media at 5-50g/l (w/v).

The preferred conifer species for the practice of the invention are *Pinus* species, especially *Pinus taeda*, *Pinus elliottii* and *Pinus radiata*. Also preferred is *Pinus strobus*. Another

20   preferred species is *Pseudotsuga menziesii*.

A genetic transformation may be carried out by a number of artificial gene transfer protocols e.g. PEG based methods, electroporation and particle delivery systems. The most preferred method of carrying out the transformations is to use bombardment techniques in

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which DNA on carrier particles is accelerated at a speed such that carrier particles are introduced into the target cells to be transformed (e.g. see US Patents 5,036,006, 5,100,792 and 5,179,022). Gold particles are the preferred carrier particles.

5   In a preferred embodiment the conifer embryogenic tissue which has been stably transformed is selected by growing embryogenic tissue on a medium containing extremely toxic levels of a selection agent. The medium is chosen so that only stably transformed embryogenic tissue grows on it and non-transformed tissue does not grow. A preferred selection agent is geneticin at 15 mg/l or higher where the tissue has been bombarded with

10   the *npt* II construct. Other selection agents may be used such as the herbicide chlorsulfuron when the cells have been bombarded with an appropriate resistant gene. Preferably the selection agent is used in the medium during the stages of somatic embryo proliferation and development until such time as an embryo axis has been established. The use of the selection agent may commence as soon as three days after bombardment with foreign genes.

15

The foreign genes to be inserted into the conifer species may include genes conferring disease resistance upon the conifers or other desirable traits. It is preferred to include within the material incorporated sequences which will increase integration frequency of the foreign gene into conifer DNA. Preferred for this purpose are agrobacterium border

20   sequences. Vector pRC 101 (Fig. 1) is an example of a vector in which there are such sequences. In preferred embodiments the DNA used to transform the conifer cells includes a viral promoter to express foreign genes in the conifer. Cauliflower mosaic virus 35S promoter is particularly preferred.

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It is preferred to incorporate into the DNA used in the transformation a visible marker to improve efficiency of gene transfer into and expression in a conifer. A preferred gene for this purpose is *uid* A gene, (also called *gus* gene from *E. coli*).

5   Also preferred is the use of the pEmu promoter to improve efficiency of gene expression in conifers.

Also preferred is the use of the Kozak consensus sequence to improve efficiency of messenger translation in conifers.

10

Once the stably transformed embryogenic tissue has been identified using the selection agent, it is preferred to retain a substantial portion of it in the vigorous juvenile state by cryopreserving it in liquid nitrogen while clonal field trials are carried out. If the clonal field trials demonstrate suitable field performance, the use of cryopreservation allows

15   recovery of the transformed embryogenic tissue from the liquid nitrogen to provide a source of the tissue from which large numbers of vigorous juvenile plants can be regenerated.

According to another aspect of the invention, there is provided conifer plants produced by the methods of the invention. The methods of the invention allow the production of large

20   number of transgenic plants from a single cell which can be allowed to multiply before being allowed to mature into embryos and subsequently be used to produce plants.

BRIEF DESCRIPTION OF THE DRAWINGS

Schematic of the plasmid vectors used in this study.

5   Figure 1   pRC 101 is a binary vector based on pJE 189 and the *gus* reporter

gene as well as the *npt* II gene are represented by solid blocks.

Figure 2   pCW 122 is a pUC derived construct. The *gus* gene is under control

of the 2x35S promoter, whereas the *npt* II gene is controlled by the

35S promoter. A Kozak consensus sequence is cloned around the

10   ATG start codon of the *gus* gene.

EXAMPLES

The following examples further illustrate the invention.

15

In the examples, the Biolistic® particle delivery system (DuPont PDS 1000 He) was used

to transform *Pinus radiata* embryogenic initials which were bombarded in an

undifferentiated state (1 - 8 celled initials). A ß-glucuronidase (*gus*) gene under the control

of an Emu promoter and an *npt* II gene under the control of the CaMV 35S promoter were

20   cloned and transferred into embryogenic tissue, using conditions which gave up to 3000

transient spots per gram fresh weight of tissue. Transfer of bombarded cells to selective

media resulted in the formation of stably transformed tissue whereas non-transformed cells

died. Geneticin resistant embryogenic tissue was recovered from several independant

transformation experiments at a frequency of 1 - 4 transformed cell lines per bombardment

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using different *Pinus radiata* clones. The tissue continued to grow on selective media with successive transfers, and this tissue, as well as mature embryos and plants regenerated from it, expressed the *gus* reporter gene. Polymerase Chain Reaction, Southern and Northern blotting were used to confirm stable integration of the *gus* and *npt* II genes into the genome

5   of *Pinus radiata,* and expression in tissue and plants. More than 150 plants from independent transformation experiments were recovered and are growing well in the greenhouse for further molecular analysis.

Example 1 describes the method for selection of the transformed tissue and generation of

10   transformed plants. Example 2 describes the verification of the transformation. Example 3 describes the experimental protocol used including culture methods, vector constructs, the transformation method and methods used to verify the transformation. Example 4 describes results with *Pinus taeda*.

15   EXAMPLE 1   SELECTION OF GENETICIN RESISTANT TISSUE AND PLANT
                          REGENERATION

To select for stably transformed *Pinus radiata* tissue, kanamycin and geneticin (G418) were originally considered as selective agents. In experiments with normal embryogenic tissue,

20   kanamycin was observed to allow growth of tissue at even very high concentrations (Hargreaves and Smith, NZ FRI unpublished data), whereas with geneticin tissue was never observed growing on concentrations higher than 15 mg/l, and even at 10 mg/l, fresh weight increment was suppressed by up to 90%.

In the transformation experiments reported below, geneticin at 15 mg/l was used as a selection agent unless otherwise noted. Within 2 - 8 weeks on selection medium following bombardment with the *npt* II gene, between 0 - 4 (mean 0.5) centres of growth of embryogenic tissue were detected per plate. These usually continued to grow following

5 transfer to fresh medium with geneticin, but several transfers were often required before adequate rates of growth were observed. Embryogenic tissue was maintained in an undifferentiated state by the addition of plant growth regulators (medium EDM6, containing 5 mg/l 2,4-D and 0.5 mg/l BAP), or allowed to undergo development to form "bullet" stage embryos on medium (EDM) without plant growth regulators. Geneticin selection was

10 maintained throughout this phase. After 6 weeks on EDM without growth regulators, tissue was transferred to Embryo Maturation Medium (EMM) containing abscisic acid at 15 mg/l[-1.] Mature somatic embryos were recovered from EMM and subsequently germinated. (Note that media used are described in Example 3 together with methods used).

15 In preliminary experiments, geneticin was used in all stages of embryo development, however we later determined that it was not necessary once "bullet" stage embryos were transferred to EMM. Germinated somatic embryos were transferred to soil as described in Australian Patent Application 60707/94. More than 150 plants from different transformation events are now growing in the GMO greenhouse.

20

EXAMPLE 2   VERIFICATION OF STABLE TRANSFORMATION

Expression of *gus* in transformed tissue. An optimisation procedure for high transformation efficiency using embryogenic tissue of *Pinus radiata* was discussed in an

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earlier paper[5]. Vectors used for bombardment were pRC 101 and pCW 122 (see Figures 1 and 2). The bombardment protocol was used for transformation and the expression of the *gus* reporter gene was analysed histochemically and fluorometrically. Stably transformed lines displayed differences in expression intensity in histochemical assays, and in order to

5 quantify results, fluorometric assays were performed (table 3). *Gus* expression also appeared to be dependent on developmental stages of the tissue. In stably transformed tissue on maintenance medium, *gus* stain intensity was relatively high, but was expressed only faintly in "bullet" stage somatic embryos. The stain was observed to be localised on the surface of needle shaped crystalline cytoplasmic inclusions in these embryos. At the time of

10 formation of cotyledons, *gus* was expressed strongly in the hypocotyl but only faintly in the cotyledons, especially towards the distal end. In the transformed plants, *gus* stain was observed only in the vascular tissue of shoots, however plants have not yet been sacrificed for systematic studies of other tissues. *Gus* expression was not observed in the vascular tissue of non-transformed plants.

15

TABLE 3

Expression of *gus* In Transgenic Embryogenic *Pinus radiata* Tissue

Pinus radiata	VECTOR	GENETICIN-RESISTANCE	GUS-activity (nmol MU/min*mg protein)
D93-199	-	-	0.08
D93-199	pRC 101	+	0.8
D93-199	pRC 101	+	1.8
D93-199	pRC 101	+	7.1
D93-199	pRC 101	+	11.1
D93-199	pRC 101	+	13.0

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Pinus radiata	VECTOR	GENETICIN-RESISTANCE	GUS-activity (nmol MU/min*mg protein)
D93-199	pRC 101	+	15.6
D93-199	pCW 122	+	43.6
A93-13	pRC 101	+	3.3
Q92-1	pRC 101	+	1.6
D93-199	pCW 122	+	64.3

PCR analysis of transformed tissue. Primers for *gus* and *npt* II were designed (see material and methods) to amplify internal fragments of 677 and 804 bp length respectively. Several different transformed embryogenic lines were analysed and the presence of *gus* and *npt* II were confirmed in all transgenic material, whereas corresponding bands were never detected in control tissue. Needles of transformed plants were tested and the presence of *gus* and *npt* II was demonstrated in this material, confirming the histological observations. In some transgenic lines which were transformed with pRC 101 and which do not express detectable amounts of ß-glucuronidase (histochemical and fluorometric analysis), the presence of the gene was confirmed by PCR.

Southern analysis. Three embryogenic lines transformed with the plasmid vector pRC 101 were tested by Southern blotting. No bands were detected in non-transgenic lines using either *gus* or *npt* II as probes. In transgenic material, at least 1 band was observed, indicating 1 or multiple integrated copies of the foreign genes in the *Pinus radiata* genome. Integrations were at different sites and no evidence for tandem arrangements could be found. Southern analysis with one of the transformed plants confirmed the transformed nature of this plant.

Northern blot analysis

A number of transgenic tissue lines were tested for the expression of *gus* and *npt* II using Northern blotting. Tissue transformed with the vector pRC 101 as well as pCW 122 showed

5   hybridisation with the *gus* and *npt* II probe at the expected site, indicating the expression of the genes in radiata tissue. No bands could be detected in non transformed tissue.

Discussion

10   Optimal conditions for transient transformation were used to stably integrate foreign genes into embryogenic tissue of *Pinus radiata*. The observed frequency of between 0 and 4 (average 0.5) stably transformed cell lines per bombardment gives us an efficient method to routinely introduce foreign genes into the important plantation species *Pinus radiata.* Kanamycin was tested as a selective agent, but growth of non transformed tissue was

15   frequently observed on high concentrations. Geneticin (G418) was found to prevent the growth of non transformed tissue at concentrations of 15mg/l. Other investigators reported the use of kanamycin as a selection agent, and noted a frequency of escapes as high as 90%.

20   We have not recorded escapes when using geneticin at 15 mg/l with *Pinus radiata*. The embryogenic cell lines which proliferate following bombardment, and which survive transfer to fresh selection medium, invariably prove to be stably transformed when subjected to molecular analysis.

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The conifer transformation protocol described by Ellis et al[6] involved bombardment of pre-cotyledonary or cotyledonary stage somatic embryos, with subsequent de-differentiation to form embryogenic tissue. The protocols which we report here are more efficient in that we are able to transform undifferentiated embryogenic tissue from the proliferation medium.

5   This is of particular benefit with *Pinus radiata* and other pines where the protocols which give rise to mature embryos require several steps, and the yield of mature somatic embryos per plate is considerably lower than that typically reported for non-transformed *Picea sp* and *Larix sp*. We have been able to bombard up to 180 plates per day of embryogenic tissue using the protocols which we describe here, and our results indicate the potential for

10   establishing about 90 stably transformed cell lines per day, given appropriate promoters and genes.

The germinated somatic embryos which we have transferred to soil have developed into plants which appear to be similar in form and growth rate to untransformed members of

15   the same clones. No disruption of development resulting from the transformation and integration of foreign genes into essential plant genes has been observed to date. A degree of accelerated maturation has been observed in some of the transformed plants, but this is also seen in occasional batches of untransformed somatic seedlings, and in micropropagated *Pinus radiata*, and is probably the result of stress in the greenhouse.

20

Plant regeneration was achieved with some, but not all of the embryogenic tissue lines transformed. Some of the lines lost their plant regeneration potential during culture on maintenance medium and did not produce mature embryos. This is not necessarily a result of the transformation per se, but is a phenomenon regularly observed in embryogenic lines

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which have been maintained for a period of 12 - 18 months (Smith and co-workers, NZ FRI unpublished data). Transformed lines may be cryopreserved in liquid nitrogen for future plant production, and plants have been regenerated from cryopreserved transformed embryogenic tissue.

5

The *npt* II gene in our experiments is under the transcriptional control of the CaMV 35S promoter, which has been shown previously not to be a high expressing promoter in *Pinus radiata* embryogenic tissue. However, its expression is strong enough in stably transformed tissue to confer resistance against the antibiotic. This finding extends the range of promoters

10   which can be used in the transformation of radiata pine.

Fluorometric *gus* assays to quantify the amount of active ß-glucuronidase confirmed histochemical observations of different levels of *gus* expression in independently transformed *Pinus radiata* tissue. One explanation for this phenomenon could be a

15   chimaeric nature of the transformed tissue with only a limited percentage of the cells being transformed. However, due to the primitive nature of the tissue used for transformation, we consider this to be unlikely. At the time of bombardment the tissue typically comprises 1 to 8 celled initials. Histochemical analysis of transiently transformed tissue 3 days after bombardment showed expression typically in single celled initials and only rarely in

20   multicelled initials, and we believe that the transformed cell lines generally arise from single cells following bombardment. Position effects or copy number effects, which are reported for other transformed plants, could also be the reason for the observed variable *gus* expression. The observation of localisation of *gus* stain on the surface of cytoplasmic crystalline inclusions at some stages of development, but not at others, for the same cell

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lines indicates that there may be variation in *gus* expression due to compartmentalisation of the *gus* protein in some tissues. Further experiments are necessary to understand this phenomenon better.

5   Southern blot analysis of several lines confirmed the transformed nature of the tissue and Northern blotting confirmed both genes from both vectors being expressed in transgenes. Problems resulting from tandem arrangements and gene distortion due to the biolistic process have not been detected and confirm the applicability of the biolistic process to conifers. In the lines tested, numbers and arrangement of integrated genes are comparable

10   to *Agrobacterium* transformation of other plants. Since *Agrobacterium* has not yet been demonstrated to be a useful gene transfer system for *Pinus* species, and since the protocol described here is reliable and efficient, we believe that our system will be the preferred option for transformation of this species.

15   Results from experiments carried out as above, but with *Pinus taeda* tissue replacing *Pinus radiata* tissue have also demonstrated stable transformation of tissue from *Pinus taeda*. This has been confirmed by growth of tissue on selective media and using histochemical and fluorometric gus assays.

20   Results from experiments with embryogenic cultures of *Pinus strobus*, *Pinus elliottii* and *Pseudotsuga menziesii* indicate that both our plant regeneration and transformation protocols are applicable to these economically important conifer species as well. We believe that the protocols described here now give us the capacity to insert into *Pinus* species and

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Pseudotsuga menziesii some commercially important traits such as disease resistance, wood durability and wood quality.

EXAMPLE 3   EXPERIMENTAL PROTOCOL

5

Tissue culture and somatic embryogenesis of *Pinus radiata*

Pinus radiata embryogenic tissue (200 mg fresh weight) was suspended on Whatman filter paper on liquid Standard Embryogenesis Medium (SEM, as Table A5 but without the agar).

10   Three days after bombardment, the tissue was transferred on the filter paper to EDM6 (EDM (Table A6) containing 5 mg/l 2,4-D and 0.5 mg/l BAP) containing 15 mg/l^{-1} geneticin. Geneticin at this concentration was shown to totally inhibit growth of normal embryogenic tissue, and even at 10 mg/l^{-1}, fresh weight growth was reduced to less than 10% of that of tissue on geneticin-free medium. Within 2 - 8 weeks on selection medium,

15   from 0 to 4 centres of growth of embryogenic tissue were detected on each plate. These usually continued to grow on transfer to fresh medium with geneticin, but several transfers were required before adequate rates of growth were observed. Embryogenic tissue was maintained in an undifferentiated state on EDM6 or allowed to undergo development to form "bullet" stage embryos on medium without plant growth regulators. Geneticin

20   selection was maintained throughout this phase. After 6 weeks on EDM without growth regulators, tissue was transferred to Embryo Maturation Medium (EMM, Table A7) containing abiscisic acid at 15 mg/l^{-1} Mature somatic embryos were recovered from EMM and subsequently germinated as described in Australian Patent Application 60707/94. In preliminary experiments, geneticin was used in all stages of embryo development, however

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we later determined that it was not necessary once "bullet" stage embryos were transferred to EMM. Germinated somatic embryos were transferred to soil as previously described in Australian Patent Application 60707/94.

5 MEDIA FOR EMBRYOGENIC TISSUE CULTURE

TABLE A1 - Major Ion Stock

Compound	Weight gm
KNO_3	14.31
$MgSO_4.7H_2O$	4.00
$CaCl_2.2H_2O$	0.25
$NaNO_3$	3.10
$NH_4H_2PO_4$	2.25

make up to 400 ml

15 **TABLE A2 - Minor Ion Stock**

Compound	Weight mg
$MnSO_4.4H_2O$	36.0
H_3BO_3	80.0
$ZnSO_4.7H_2O$	250.0
KI	10.0
$CuSO_4.5H_2O$	24.0
$Na_2MoO_4.2H_2O$	2.0
$CoCl_2.6H_2O$	2.0

make up to 200 ml

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TABLE A3 - Iron stock - to make 1 litre

FeSO$_4$.7H$_2$O	1.5 gm
Na$_2$EDTA	2.0 gm

5   **TABLE A4 - Vitamin stock - to make 1 litre**

Thiamine HCl	0.5 gm
Nicotinic acid	0.5 gm
Pyridoxine HCl	0.05 gm

10   **Table A5 - Standard Embryogenesis Medium (embryogenic tissue maintenance medium)**

	per litre of medium
Major ion stock	40 ml
Minor ion stock	20 ml
Iron chelate stock	20 ml
Vitamin stock	10 ml
Inositol	1.0 gm
Sucrose	30.0 gm
Difco Bacto agar	8.0 gm

20

pH adjust to 5.6-5.8 before addition of agar and autoclaving

add the following filter sterilised amino acids after autoclaving:

major amino acids	milligram per litre

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glutamine	110
asparagine	105
arginine	35
minor amino acids stock	2 ml per litre

5

Table A5b - Minor amino acid stock

amino acid	gm
citrulline	1.58
ornithine	1.52
lysine	1.10
alanine	0.8
proline	0.7

10 (lysine row)

3.1   Make up to 800 ml with double distilled water.

15   3.2   Dispense into 40 ml aliquots.

3.3   Freeze immediately, store frozen, and thaw only on day of use.

3.4   Adjust pH to 5.6-5.8 and filter sterilise before use.

Table A6 - Embryo Development Medium

20

	per litre of medium
Major ion stock	40 ml
Minor ion stock	20 ml
Iron chelate stock	20 ml
Vitamin stock	10 ml

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Inositol	1.0 gm
Sucrose	30.0 gm
Kelco Gelrite	3.0 gm

5   pH adjust to 5.6-5.8 before addition of agar and autoclaving.

Add the following filter sterilised amino acids after autoclaving.

major amino acids	milligram per litre
glutamine	550
10 asparagine	510
arginine	175
minor amino acids stock	10 ml per litre
(as per Table A5b)	

15   **Table A7 - Embryo Maturation medium (EMM)**

To make one litre of medium

Step 1

Major ion stock	40 ml
20 Minor ion stock	20 ml
Iron chelate stock	20 ml
Vitamin stock	10 ml
Inositol	1 gm
Sucrose	30 gm

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Dissolve in double distilled water, and adjust volume to allow for addition of filter sterilised

components.

Adjust pH to 5.7

5

Add Gelrite 3 gm per 500 ml flask (6 gm per litre) then add pH adjusted liquid.

Autoclave.

Step 2

10   Dissolve with heating to give final volume of 50 ml

Minor amino acid stock   40 ml

Glutamine   7.3 gm

Asparagine   2.1 gm

Arginine   0.7 gm

15   Abscisic Acid   15 mg

(dissolve in 1N NaOH)

Filter sterilise and add to autoclaved medium

20   **Vector constructs.** Cloning of vectors for transformation experiments was according to

standard protocols[7] . Plasmid pRC 101 (figure 1) is a binary vector derived from pJE 189[8],

containing the *gus* reporter gene under control of the Emu promoter[9] and *nos* terminator.

The *npt* II gene has a 35S promoter and CaMV terminator. Plasmid pCW 122 is a pUC

derivative with the *gus* gene under transcriptional control of the 2x35S promoter. A Kozak

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consensus sequence is located around the ATG start codon[10]. The *npt* II gene is controlled by the 35S promoter.

Biolistic transformation. DNA for bombardment experiments was isolated using the

5   Promega Wizard prep kit. The DuPont Biolistic® particle delivery device (PDS 1000He) was used for all transformation experiments. Gold particles (Aldrich Chemicals, Milwaukee, USA) were prepared as described by Sanford *et al*[11] and the following physical bombardment conditions were used for transformation: Gap 6 mm; Macrocarrier travel distance 11 mm; Microcarrier travel distance 9 cm; rupture disc pressure 1350psi; vacuum

10   in chamber 25 mm Hg. Embryogenic tissue was suspended in liquid SEM medium and plated on a 6 cm Whatman filter paper on solid EDM6 medium (200 mg tissue/ml) one day prior to bombardment, and dried in the laminar flow overnight.

Histochemical and fluorometric analysis of *gus* expression. For histochemical staining,

15   the tissue was flooded with a *gus* staining solution[5] and incubated for 48 hours at 37°C. Blue cells and clusters of blue cells were counted using a Leitz Stereo microscope. Fluorometric assays were performed using a TK100 fluorometer (Hoefer). ß-glucuronidase was extracted from plant tissue with the extraction buffer (Gallagher, 1992)[12] and total protein was measured using the BioRad protein assay. Fluorometry was according to a

20   protocol by Gallagher (1992)[12]. Results are the mean of 4 individual assays and ß-glucuronidase activity was calculated.

Isolation of genomic DNA from transformed tissue of *Pinus radiata*. Total DNA from transformed and non transformed tissue for PCR and Southern analysis was isolated using

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a CTAB method: 3g of tissue was extracted in 20ml of CTAB at 60°C using mortar and

pestle (for Southerns) or in smaller amounts in eppendorf tubes (for PCR), and

subsequently incubated for 30 min at 60°C. Chloroform/Isoamyl alcohol (24:1) was added,

mixed and centrifuged for 5 min. The top fraction was removed and the process repeated.

5   20ml of isopropanol (-20°C) was subsequently added to the top fraction, mixed and left on

ice for 20 min. After centrifugation at 4500 rpm for 20 min, the supernatant was discarded

and the pellet resuspended in 0.5ml 1M NaCl over night. The solution was then heated for

10 min at 37°C), spun for 5 min and the supernatant mixed with 0.5 ml isopropanol (-

20°C). After incubation on ice for 20 min, the preparation was spun for 15 min, the pellet

10 washed with 70% ethanol followed by 95% ethanol. The supernatant was saved and

precipitated again. The pellets were combined and resuspended in 0.5ml TE. Quality and

concentration of DNA were tested by spectrophotometry and gel electrophoresis. DNA was

isolated from needles of *Pinus radiata* using a similar method, but the needles were ground

in liquid nitrogen.

15

Isolation of RNA and Northern analysis

Total RNA of embryogenic *Pinus radiata* tissue was isolated according to the protocol

described by Chang et al.[13]. RNA was separated in denaturing agarose gels (1%, 6%

formaldehyde, $30\mu g$ RNA / lane), and transferred to nylon membranes (Hybond N+) via

20 capillary blotting using standard protocols ([19]). RNA was subsequently hybridised to

random primer labelled DNA (Pharmacia Ready to Go) in 1xPipes, 10xDenhardt, 0,1%SDS

and $100\mu g/ml$ salmon sperm DNA at 60°C. Blots were washed 2x20 min in 0,2xSSC at

62°C, and exposed to Kodak X-Omat, AR film.

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PCR analysis.

Primers for amplification of *gus* and *npt* II were as follows: Gus: CTGTAGAAACCCCAACCCGTG and CATTACGCTGCGATGGATTCC and for *npt* II: GAGGCTATTCGGCTATGACT and AATCTCGTGATGGCAGGTTG: For PCR, 100 ng

5 of target DNA was used in a standard cocktail as follows: 0.4 μl primers, 1u Taq, 2mM MgCl$_2$, 1xbuffer (Boehringer). Prior to cycling, the sample was heated to 93°C for 3 min. Cycling was 30sec melting at 94°C, 30sec annealing at 65°C, 30sec elongation at 72°C. After PCR, the products were analysed on an electrophoresis gel. Magnesium concentrations and melting temperatures were optimised to produce one distinct target band and reduce or

10 eliminate the production of non specific bands. Controls with vector DNA and non transformed *Pinus radiata* tissue were always included.

Southern blotting. DNA for Southern blotting was digested with restriction enzymes over night according to standard protocols[7]. Thirty μg of DNA per lane were used in gel

15 electrophoresis and blotted to Nylon membranes (Hybond N+). Probes (*gus* and *npt* II) were labelled using the Pharmacia Ready to Go kit and Southerns were performed.

EXAMPLE 4   STABLE TRANSFORMATION OF PINUS TAEDA

20 Transformation of *Pinus taeda* was carried out as described in Examples 1-3 for *Pinus radiata*. That the transformed cells were also stably transformed was confirmed by growth of tissue on the selective media and by using histochemical and fluorometric *gus* assays. 20 lines of transgenic *Pinus taeda* were recovered from 6 independent clones and analysed in histochemical *gus* experiments. All transgenic lines were *gus* positive, whereas

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nontransformed lines never showed any *gus* expression. All transgenic lines were geneticin

resistant, whereas all nontransgenic controls died when exposed to the antibiotic. An

example of fluorometrically analysed *gus* expression is shown in Table 4.

5   TABLE 4

Expression of gus in Transgenic Embryogenic *Pinus taeda* tissue:

Pinus taeda	Vector	Geneticin Resistance	GUS-activity (nmol MU/min˙mg protein
94.4	-	-	0.04
94.4	pRC 101	+	2.6

10

Aspects of the invention have been described by way of example only and it should be

appreciated that modifications and additions thereto may be made without departing from

the scope of the invention.

15   **References**

1.   Huang, Y., Diner, A.M. and Karnosky, D.F. 1991. *Agrobacterium rhizogenes*-
      mediated genetic transformation and regeneration of a conifer: *Larix decidua*. In
      Vitro Dell. Dev. Biol. 27:201-207.

20
2.   Wilson, S.M., Thorpe, T.A. and Moloney, M.M. 1989. PEG - mediated expression
      of GUS and CAT genes in protoplasts from embryogenic suspension cultures of
      Picea glauca. Plant Cell Reports 7:704-707.

25   3.   Bekkaouni, F., Pilon, M., Raju D.S.S., Crosby, W.L. and Dunstan, D.I.. 1988.
      Transient gene expression in electroporated *Picea glauca* protoplasts. Plant Cell
      Report 7:481-484.

4.   Tautorus, T.E., Bekkaouni, F., Pilon, M., Datla, R.S.S., Crosby, W.L., Fowke, L.C.
30   and Dunstan, D.I. 1989. Factors affecting transient gene expression in electroporated
      black spruce (*Picea mariana*) and jack pine (*Pinus banksiana*) protoplasts. Theor.
      Appl. Genet. 78:531-536.

- 26 -

SUBSTITUTE SHEET

5.   Walter, C., Smith, D.R., Connett, M.B., Grace, L. and White, D.W.R. 1994. A biolistic® approach for the transfer and expression of a *gus*A reporter gene in embyrogenic cultures of *Pinus radiata*. Plant Cell Reports 14:69-74.

6.   Ellis, D.D., McCabe, D.E., McInnis, S., Ramachandran, R., Russell, D.R.,Wallace, K.M., Martinell, B.J., Roberts, D.R., Raffa, K.F. and McCown, B.H. 1993. Stable transformation of *Picea glauca* by particle accelleration. Bio/Tech. 11:84-89.

7.   Sambrook, J., Fritsch, E.F., Maniatis, T. 1989. Molecular Cloning - A Laboratory Manual, C. Nolan (eds). Cold Spring Harbor Laboratory Press.

8.   Jones, J.D.G., Shlumukov, L., Carland, F., English, J., Scofield, S.R., Bishop, G.J. and Harrison, K. 1992. Effective vectors for transformation, expression of heterologous genes, and assaying transposon excision in transgenic plants. Transgenic Research 1:285-297.

9.   Last, D.I., Brettell, R.I.S., Chamberlain, A.M., Chaudhury, A.M., Larkin, P.J., Marsh, E.L., Peacock, W.J. and Dennis, E.S. 1991. pEmu: an improved promoter for gene expression in cereal cells. Theor. Appl. Genet. **81:** 581-588.

10. Kozak, M. 1989. The scanning model for translation: An update. The J. Cell Biol. **109:** 229-241.

11. Sanford, J.C., Smith, F.D. and Russell, J.A. 1993. Optimizing the biolistic process for different biological applications. Meth. Enzymol. **217:**483-509.

12. Gallagher, S.R. 1992. Quantitation of *gus* activity by fluorometry. *In* GUS protocols: Using the GUS gene as a reporter gene expression. Academic Press, Inc.

13. Chang, S., J. Puryear and J. Cairney (1993): A simple and efficient method for isolating RNA from pine trees. Plant Molecular Biology Reporter 11 (2), 113-116.

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CLAIMS

1.   A method for stably inserting foreign genes into conifer cells wherein the cells to be transformed are in an undifferentiated state such that embryos are likely to regenerate from a single cell.

2.   A method for stably inserting foreign genes into conifer cells wherein conifer embryogenic tissue is transformed and the transformed tissue is subjected to extremely toxic levels of selection agent after insertion of foreign genes wherein the foreign genes include genes protective against the selection agent and wherein only stably transformed embryogenic tissue grows on the medium, and nontransformed tissue does not grow.

3.   A method of claim 1 wherein the transformed cells are subjected to extremely toxic levels of selection agent after insertion of foreign genes wherein the foreign genes include genes protective against the selection agent and wherein only stably transformed embryogenic tissue grows on the medium, and nontransformed tissue does not grow.

4.   A method of claim 3 wherein the embryogenic tissue is allowed to mature to yield mature embryos which are germinated to produce transgenic plants.

5.   A method of claim 3 wherein the transformation comprises an artificial gene transfer step.

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6.   A method of claim 5 wherein said artificial gene transfer step is selected from PEG-based methods, electroporation and particle delivery methods.

7.   A method of claim 6 wherein said artificial gene transfer method involves a bombardment technique in which DNA on carrier particles is accelerated at a speed such that carrier particles are introduced into the target cells to be transformed.

8.   A method of claim 7 wherein said carrier particles are gold particles.

9.   A method of claim 3 wherein embryogenic tissue is grown on a medium comprising:

ION	Concentration (mmoles/l)
NO_3	8-27
NH_4	0.95-3
Ca	0.08-0.25
Fe	0.05-0.15
Na	1.9-5.75
Zn	0.045-0.135
Cu	$4.5 \times 10^{-3} - 1.5 \times 10^{-2}$
Mg	0.8-2.5

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10.   A method according to claim 9 wherein said medium comprises:

ION	Concentration (mmoles/l)
NO_3	13-23
NH_4	1.5-2.5
Ca	0.12-0.21
Fe	0.07-0.13
Na	2.9-4.9
Zn	0.06-0.12
Cu	7×10^{-3}-1.2×10^{-2}
Mg	1.2-2.0

11.   A method of claim 10 wherein the medium comprises:

ION	Concentration (mmoles/l)
NO_3	about 17.8
NH_4	about 1.96
Ca	about 0.17
Fe	about 0.10
Na	about 3.85
Zn	about 0.09
Cu	about 9.61×10^{-3}
Mg	about 1.62

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12. A method as claimed in claim 11 wherein embryogenic tissue is maintained, or allowed to develop or mature on a medium selected from (a) Standard Embryogenesis Medium as defined in Table A5 and (b) Embryo Development Medium as defined in Table A6 containing 2,4-D and/or 6-benzylaminopurine.

5

13. A method of claim 12 wherein the medium is Embryo Development Medium containing 1-5 mg/l 2,4-D and 0.1-0.9 mg/l 6-benzylaminopurine.

14. A method of claim 3 wherein the selection agent is a herbicide and wherein the

10 tissue has been bombarded with a construct containing an appropriate resistance gene.

15. A method as claimed in claim 3 wherein the selection agent is geneticin at 15 mg/l or higher, wherein the tissue has been bombarded with the *npt* II construct.

15 16. A method as claimed in claim 3 wherein the selection agent is used in the medium during the stages of somatic embryo proliferation and development until such time as an embryo axis has been established.

17. A method as claimed in claim 7 wherein *Agrobacterium* border sequences are

20 included in the DNA on the carrier particles accelerated into the target cells.

18. A method as claimed in claim 8 wherein the DNA includes a viral promoter to express foreign genes in the conifer.

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19.   A method of claim 18 wherein said viral promoter is cauliflower mosiac virus 35S

promoter.

20.   A method of claim 7 wherein a visible marker is used to improve efficiency of gene

5   transfer into and expression in a conifer.

21.   A method of claim 19 wherein said gene is the *gus* gene from *E. coli*.

22.   A method of claim 7 wherein the DNA includes a pEmu promoter to promote

10   efficiency of gene transfer into and expression in conifers.

23.   A method of claim 7 wherein said DNA includes the Kozak consensus sequence,

to improve efficiency of messenger translation in conifers.

15   24.   A method of claim 4 wherein a substantial proportion of the transformed

embryogenic tissue is cryopreserved in liquid nitrogen while clonal field trials are carried

out on a portion of the transformed embryogenic tissue.

25.   A method of claim 4 wherein embryogenic tissue is cryopreserved in liquid nitrogen

20   and is subsequently recovered from that and subsequently matured into embryos which are

subsequently used to produce plants.

26.   A method as claimed in claim 3 wherein said conifer belongs to the genus *Pinus*.

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27.   A method as claimed in claim 3 wherein said conifer is selected from *Pinus radiata*, *Pinus elliottii*, *Pinus taeda*, *Pinus strobus* and *Pseudotsuga menziesii*.

28.   A method according to claim 27 wherein said conifer is *Pinus taeda* or *Pinus*

5   *radiata*.

29.   A method according to claim 28 wherein said conifer is *Pinus radiata*.

30.   A genetically transformed coniferous plant produced by a method of claim 4.
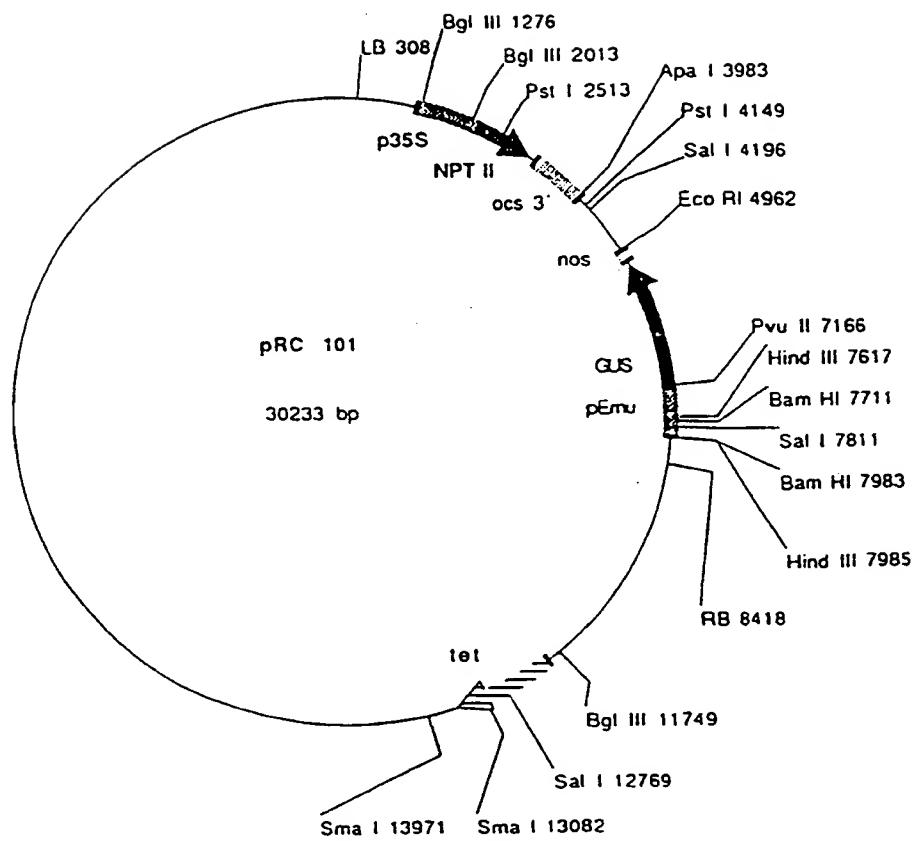
10

31.   Progeny of a coniferous plant of claim 30.

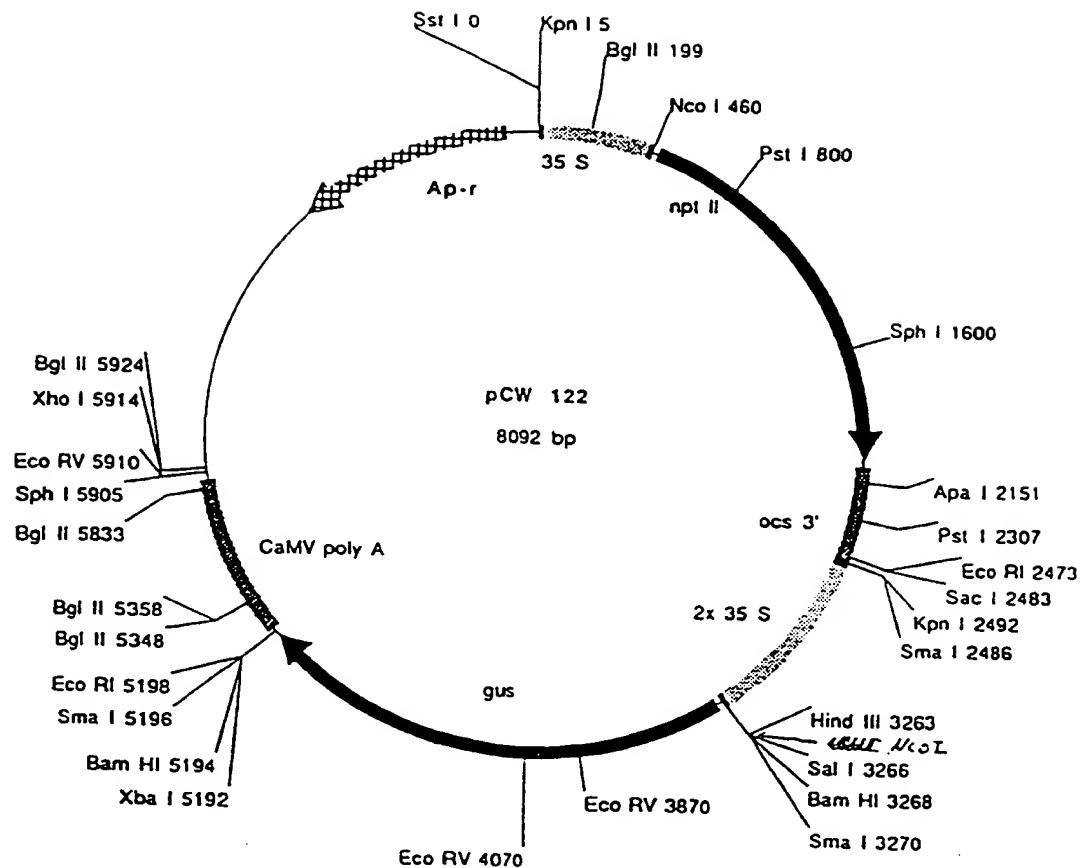32.   A vegetatively-propagated plant derived from a coniferous plant of claim 30.

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FIG 1

2/2
FIG 2

Sst I 0
Kpn I 5
Bgl II 199
Nco I 460
35 S
Ap-r
npt II
Pst I 800
Sph I 1600

pCW 122
8092 bp

Bgl II 5924
Xho I 5914
Eco RV 5910
Sph I 5905
Bgl II 5833

CaMV poly A

ocs 3'
Apa I 2151
Pst I 2307
Eco RI 2473
Sac I 2483
Kpn I 2492
Sma I 2486

2x 35 S

Bgl II 5358
Bgl II 5348
Eco RI 5198
Sma I 5196
Bam HI 5194
Xba I 5192

gus

Hind III 3263
Sal I 3266
Bam HI 3268
Sma I 3270

Eco RV 3870
Eco RV 4070

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]:   C12N 15/82, 15/63, 15/87, 5/04, A01H 7/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
WPAT and CHEM ABS
See details in electronic database box below.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DERWENT WPAT, CHEM ABS DATABASES; KEYWORDS; CONIFER:, TRANSFORM:, EMBRYOGENIC:() TISSUE, TISSUE() CULTURE #, PINUS, PSEUDOTSUGA, BIOLISTIC, EMBRYO? UNDIFFERENT?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Plant Cell Reports (1994), Vol 14, No. 2/3, pg 69 - 74, (Walter C et al) "A BIOLISTIC APPROACH FOR THE TRANSFER AND EXPRESSION OF A gus A REPORTER GENE IN EMBRYOGENIC CULTURES OF PINUS RADIATA	1-8,14,16,18-23, <u>26-32</u>
Y		9-13
Y	AU 60707/94 (NEW ZEALAND FOREST RESEARCH INSTITUTE LTD), PUBLISHED 27 October 1994. See claims 1,2, and 4	9-13
PX	Forest Science (1995), Vol. 44, pg 183-195, (Newton. R.J. et al), "SOMATIC EMBRYOGENESIS IN SLASH PINE (Pinus elliotii Engelm)"	1,2,3,5,6,7,8,18,19,20, 21,22,26,27.

☐ Further documents are listed in the continuation of Box C   ☐ See patent family annex

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" earlier document but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report 5 DEC 1996
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929	Authorized officer MARK DONAGHEY Telephone No.: (06) 283 2156

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C (Continuation)	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Plant Cell Reports (1993), vol. 12, No. 4, pg 189-193, (Charest P.J. et al), "MICROPROJECTILE-DNA DELIVERY IN CONIFER SPECIES: FACTORS AFFECTING ASSESSMENT OF TRANSIENT GENE EXPRESSION USING THE β-GLUCURONIDASE REPORTER GENE".	1-3,5,6,7,8,18, 19,20,21,26, 27,30-32.
X	US 5122466 (NORTH CAROLINA STATE UNIVERSITY), published 16 June 1992.	1,2,3,5-8,14, 17,18,20,21, 26-32
X	Canadian Journal of Forest research (1992), vol. 22, No. 12, PG 2014-2018(Campbell M.A. et al) "EXPRESSION OF LUCIFERASE AN β-GLUCURONIDASE IN PINUS RADIATA SUSPENSION CELLS USING ELECTROPORATION AND PARTICLE BOMBARDMENT"	1
A	Canadian Journal of Forest research (1992) vol. 22, No. 7, pg 993-996. (Loopstra, C.A. et al) "TRANSIENT GENE EXPRESSION IN DIFFERENTIATING PINE WOOD USING MICROPROJECTILE BOMBARDMENT"	
A	Plant Science: an International Journal of Experimental Plant Biology. (1986), vol. 58, No. 1, (Gupta. P.K. et al), SOMATIC PROEMBRYO FORMATION AND TRANSIENT EXPRESSION OF A LUCIFERASE GENE IN DOUGLAS FIR AND LOBLOLLY PINE PROTOPLASTS"	
X	Biotechnology (1993), vol. 11 No. 1, pg 84-89, (Ellis, D.D. et al) "STABLE TRANSFORMATION OF Picea glauca by PARTICLE ACCELERATION".	1-8,18,19,20. 21,30-32.

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
AU	94/60707	BR	950110	WO	951214	ZA	950103

END OF ANNEX